Changes in phytochemical composition, bioactivity and *in vitro* digestibility of Guayusa leaves (*Ilex guayusa* Loes.) in different ripening stages

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ABSTRACT

BACKGROUND: Guayusa (*Ilex guayusa* Loes.) leaves, native of the Ecuadorian amazon, are popularly used for preparing teas. This study aimed to assess the influence of leaf age on the phenolic compounds and carotenoids, and the bioactivity and digestibility (*in vitro*) of aqueous and hydroalcoholic leaf extracts.

RESULTS: In total, 14 phenolic compounds were identified and quantified. Chlorogenic acid and quercetin-3-*O*-hexose were the main representatives of the hydroxycinnamic acids and flavonols, respectively. Seven carotenoids were quantified, lutein was the main compound. Ripening affected significantly phenolic content, but there was no significant difference on carotenoid content. Antioxidant capacity, measured by $DPPH^{\cdot}$ method, was also significantly affected by leaf age. The measurement of *in vitro* digestibility showed a decrease in the phenolic content (59%), as well as antioxidant capacity, measured by $ABTS^{\cdot+}$ method, in comparison with initial conditions of the guayusa infusion. Antibacterial and anti-inflammatory activities were assayed with young leaves due to its higher phenolic contents. Guayusa did not show any antibacterial activity against *E. coli* ATCC 25922 or *S. aureus* ATCC 25923. Finally, the hydroalcoholic and aqueous extracts exhibited a high *in vitro* anti-inflammatory activity (>65%).

CONCLUSION: Guayusa young leaves have potential applications as functional ingredient for food and pharmaceutical industry.

Keywords: *Ilex guayusa* Loes., ripening, phenolic compounds, carotenoids, *in vitro* digestibility, hyaluronidase inhibition.

INTRODUCTION

Nowadays, there is a growing tendency to study bioactive compounds (i.e. polyphenols and carotenoids) of natural origin, mainly from plants. According to Slavin and Lloyd[1], this interest is mainly due to the beneficial effects of plant-derived bioactive compounds on human health. In this context, South America has a wealth of underexploited native plants of great interest for food technology, science and industrial applications, with many nutritional and functional properties yet to be uncovered.

Guayusa or Wayusa (*Ilex guayusa* Loes.) is a domesticated native plant of the Amazon that grows in southern Colombia, Ecuador and northern Peru. This plant has been consumed ancestrally by different ethnic groups and indigenous nationalities, mainly Jívaros, for its medicinal properties.[2] Studies have demonstrated the importance of guayusa within the ancestral knowledge for its stimulating and purgative capacity. [3]

One of the major bioactive groups of constituents of *Ilex* spp. are phenolic compounds, widely studied because of their health-promoter activities. [4, 5] In general, phytochemicals are the responsible of the bioactive properties of vegetables, playing an important role on health.[6] Indeed, the experiences of traditional therapy, such as preventing lung and liver diseases, have shown that species of the genus *Ilex* could be potential remedies for the modern diseases.[7]

On the other hand, several biochemical reactions occur during ripening, affecting the levels of phytochemicals which are responsible of the characteristics of mature fruit and plants. Throughout senescence, there is an increment in the formation of reactive oxygen species (ROS) and it is possible that secondary metabolism may be activated in order to continue cell repair mechanisms depending if secondary metabolites are needed in the cell repair process or not.[8] Blum-Silva et al.[9], demonstrated that the content of total phenolic compounds on yerba mate (*Ilex paraguariensis*), a plant related with

guayusa[7], is higher at early stages of maturity and gradually decreases with ripening. This could give an indication on plant diversity in terms of phytochemical composition, which is relevant for its future use as a raw material for the production of bioactive interesting compounds.

Although the benefits of genus *Ilex* are well established through numerous studies, there is no evidence of how the ripening stage could influence the content of bioactive components (e.g., polyphenols and carotenoids) and its properties in *Ilex guayusa* Loes.

Hence, the purposes of this study were:

1. To determine the effect of ripening on phytochemical composition (polyphenols and carotenoids) and *in vitro* antioxidant capacity of guayusa leaves.
2. To evaluate the stability of phenolic compounds under simulated gastrointestinal conditions and associated changes in antioxidant capacity.
3. To assess the antibacterial and *in vitro* anti-inflammatory properties of the guayusa leaves with higher phenolic contents.

MATERIALS AND METHODS

Plant material

Plants of guayusa reach the harvesting maturity at 4 years of age, then, leaves must be collected up to twice a year, every 4 to 6 months.[10, 11] Consequently, for the purposes of this study, the leaves were selected in their initial stage of maturation (2-month old) and when they were ready to harvest (6-months old). Leaves were collected in Pastaza province, North West of Ecuador, and stored at -18 °C until freeze-drying in a lyophilizer (Stokes, Germany). After drying, the size of the leaves was reduced to 4mm particles, using a hammer mill Condux D6451 (Karl Kolb – Frankfurt, Germany).

Standards, chemicals and solvents

The standards gallic acid, 5-*O*-caffeolyl-quinic acid, quercetin 3-*O*-rutinoside. β-carotene was purchased from Sigma-Aldrich (Steinheim, Germany). Violaxanthin, α-carotene and lutein were isolated from natural sources by classical chromatographic techniques.[12]

Monobasic sodium phosphate, dibasic sodium phosphate, 2,2-diphenyl-1-picrylhidracyl radical ($DPPH^{\cdot}$), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\cdot+}$), 2,2′-azobis(2- methylpropionamidine) dihydrochloride (AAPH), Folin Ciocalteu's reagent, fluorescein (free acid), pepsin porcine, pancreatin, bile salts, hyaluronidase and indomethacin were obtained from Sigma–Aldrich (Steinheim, Germany), while 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka Chemika (Neu-Ulm, Switzerland). Bovine serum albumin was purchased from Gibco (New York, USA).

Total phenolics content (TPC)

The total phenolic content was determined following the Folin-Ciocalteau method.[13] 0.2 g of young and mature freeze-dried leaves were extracted with 10 mL of acetone (70%) in an ultrasonic bath (Branson 2510, Branson Ultrasonic Cleaner, USA), during 30 min and shaken with a magnetic stirrer during 20 min at room temperature; finally, the extracts were filtered under vacuum and stored at -18 °C. 500 μL of the extracts, were dissolved with 2.5 mL of Folin-Ciocalteau reagent (10% v v^{-1}). The mixture was shaked with a vortex and allowed to stand for 2 min before adding 2 mL of sodium carbonate solution at 75 g L^{-1}. After 15 minutes of incubation at 50 °C, the absorbance of the solutions was measured at 760 nm on a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). For the quantification, a calibration curve of gallic acid (10-100 ppm) was

prepared under the same conditions. The results were expressed as g gallic acid equivalents (GAE) Kg^{-1} DW (dried weight).

Identification of phenolic compounds by HPLC–DAD–ESI/MS^n and quantification by HPLC–DAD

The extraction and analysis of phenolic compounds were performed using the method of Gironés-Vilaplana et al.[14] In relation to the identification, it was carried out by HPLC–DAD–ESI/MS^n analyses, using an Agilent HPLC 1100 series model equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by Chem-Station software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface, and was controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were 350 ºC and 4 kV, for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 65.0 psi and 11 L min^{-1}, respectively. The full-scan mass covered a range of 100 to 1200 m/z. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. MS data were acquired in the negative ionization mode. The MS^n was done in the automatic mode on the more abundant fragment ion in MS (n -1).

For the quantification, the HPLC-DAD system (Agilent 1220-Infinity LC) was used. Compounds separation was carried out using a Luna C18 column (25 cm x 0.46 cm, 5 µm particle size) (Phenomenex, Macclesfield, UK). The individual phenolic compounds were tentatively identified following their characteristic UV-Visible spectra, order of elution (Retention time (Rt)), and compared with standards previously established in the

HPLC-DAD–ESI-MS^n method. Hydroxycinnamic acids were quantified using chlorogenic acid (5-*O*-caffeolyl-quinic acid) as a standard at 330 nm, and flavonols using rutin (quercetin 3-*O*-rutinoside) as a standard at 360 nm. All the samples were extracted in triplicate and injected three times. The results were expressed as g Kg^{-1} DW.

Identification and quantification of carotenoid compounds by Rapid Resolution Liquid Chromatography (RRLC)

The extraction, saponification and analyses of carotenoids were carried out according to the method described by Stinco et al.[15] The extracts were saponified to eliminate the chlorophylls. The saponification conditions were: methanolic KOH solution (30 g 100 mL^{-1}) for 1 h under dim light at room temperature. Then, samples were washed with water in order to remove any trace of the alkaline solution. The colored dichloromethane extracts obtained were concentrated to dryness in a rotary evaporator at temperature below 30 □C and dissolved in 100 □L of ethyl acetate prior to their injection in the RRLC system. All the samples were extracted in triplicate. The identification was made by comparison of their chromatographic and UV–vis spectroscopic characteristics with those of standards. Meanwhile, the quantification was carried out by external calibration from the chromatographic peak areas at 450 nm. The results were expressed as □g g^{-1} DW.

Antioxidant capacity

$DPPH^{\cdot}$ method

The 2,2-diphenyl-1-picrylhydrazyl radical has a deep purple color and shows a strong absorption at a wavelength of 515 nm. The reactions of the antioxidants with DPPH are based on the electron transfer that allows the use of analytical methods for monitoring the color change from purple to yellow. $DPPH^{\cdot}$ test was performed according to the

method described by Mena et al.[16] Extracts were prepared with 3% formic acid in methanol (1:4 w v^{-1}) for 24 h at 4 °C. Then, the extracts were filtered, the methanol was removed with a rotary evaporator (35 °C), and the residues redissolved in acidified water (3% formic acid). The reaction was started by adding 2 µL of the corresponding diluted sample to the well containing 250 µL of 6 × 10^{-5} mol L^{-1} methanol $DPPH^{\cdot}$ solution. The decrease in absorbance was determined at 515 nm at 0 min and after 50 min of reaction with $DPPH^{\cdot}$.

Oxygen radical absorbance capacity (ORAC) method

The ORAC assay was performed according to Ou et al.[17] Briefly, the reaction was carried out at 37 ºC in 10 mM phosphate buffer (pH 7.4) and the final assay mixture (200 µL) contained fluorescein (1 □M), 2,2′-azobis(2-methyl-propionamidine)-dihydrochloride (250 mM) and antioxidant (Trolox [10 - 200 □M] or guayusa samples [at different concentrations]).

$DPPH^{\cdot}$ and ORAC assays were performed using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite® M200 microplate reader (Tecan, Grödig, Austria). The results were expressed as mmol Trolox $100g^{-1}$ DW.

$ABTS^{\cdot+}$ method

The method proposed by Re et al.[18], was applied to evaluate the free radical scavenging capacity. This method is based on the formation of a blue/green radical cation by the oxidation of 7 mM $ABTS^{\cdot+}$ with 2.45 mM potassium persulfate. The mixture was allowed to stand in the dark at room temperature for 12 to 16 h before use. Addition of antioxidants to the radical cation reduced the $ABTS^{\cdot+}$ depending on the antioxidant capacity, the concentration of the antioxidant, and the duration of the reaction. Therefore, the radical cation solution was freshly prepared on the day of analysis by

diluting the stock solution with pH 7.4 phosphate buffer to an absorbance of 0.70 ± 0.02 at 734 nm. 10 µL Trolox (0 to 2.5 µM) were added to 990 µL of the radical cation solution and the absorbance at 732 nm was measured to obtain a calibration curve at the incubation time optimized. This was compared with a blank where 10 µL of the antioxidant were substituted by a phosphate buffer at pH 7.4. The results were expressed as mmol Trolox $100g^{-1}$ DW.

In vitro digestibility

Guayusa infusion preparation

The infusion was obtained by the traditional preparation and consumption manner used by Amazonian communities[19], which uses old leaves because young ones disintegrate during the infusion, according to information provided by the leaf suppliers. Therefore, 2 g of freeze-dried old leaves were mixed with 240 mL of distilled water and warmed up at to 92 °C for 5 minutes, to avoid boiling, and after 3-5 min. rest, the infusion was filtered under vacuum.

In vitro digestibility of polyphenols

In vitro digestibility was performed using the method of Tavares et al.[20] and Ryan et al.[21], with slight modifications. To mimic oral phase, the original infusion (22 mL) was adjusted to pH 7 with 6 mL of artificial saliva (NaCl 6.2 g L^{-1}, KCl 2.2 g L^{-1}, $CaCl_2$ 0.22 g L^{-1} and $NaHCO_3$ 1.2 g L^{-1}) and incubated during 2 min at 37 °C with a shaking frequency of 100 oscillations min^{-1}. Aliquots (4 mL) of the post-oral phase were removed and frozen at -18 °C. The pH of the remainder infusion was adjusted to 2 with HCl 5 M, and subjected to incubation in a water bath (Precision Scientific model 25, Chicago, USA) at 37 °C for 2 h under shaking of 100 oscillations min^{-1} in the presence of 1.4 mL of stomach solution (20 mg of porcine pepsin and 2 mL of 0.1 M HCl). Four mL of the stomach-digested infusion were removed and frozen at -18 °C after

mimicking the stomachic phase. Then, the pH of the infusion was adjusted to 6 with $NaHCO_3$ (2g 100 mL^{-1}) and 4.5 mL of intestinal solution (18 mg of pancreatin with 112.5 mg of bile salts dissolved in 4.5 mL of $NaHCO_3$ 1N). After this, the pH was adjusted to 7 with $NaHCO_3$ 1N and incubated for 2 h under constant shaking of 100 oscillations min^{-1} at 37 °C. After that time, 4 mL was separated and frozen at -18 °C. For the simulation of the intestinal phase, the pH was adjusted to 7.2 with NaOH 0.5 M and centrifuged at 2500 g for 60 min at 4 °C. Finally, 4 mL was withdrawn and frozen at -18 °C. This experiment was carried out by triplicate.

Antibacterial activity assay

Extract preparation

The modified protocol proposed by Guendouze-Bouchefa et al.[22], was applied to prepare the extacts. Aqueous and hydroalcoholic extracts (water:ethanol, 80:20 v v^{-1}) were prepared with 10 g of freeze – dried young guayusa leaves, since they showed higher phenolic content[9], in 150 mL of extractant, shaken for 15 min at 15 rpm with a magnetic shaker (MSH, Boeco, Germany) and filtered under vacuum.

Microorganisms

Bacterial strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used for the screening of antibacterial activity. The cryo-stocks of each strain were prepared in Trypticase Soy Broth (TSB) (Merck, Germany) with 10% glycerol at concentrations of 5 x 10^6 Colony-forming-unit per mL (CFU mL^{-1}) for *E. coli* and 5 x 10^6 CFU mL^{-1} for *S. aureus*. The inoculum concentrations for the experiment were: *E. coli* 5 x 10^4 CFU mL^{-1} and *S. aureus* 5 x 10^3 CFU mL^{-1}.

Assessment of the antibacterial activity

Antibacterial activity of the extracts was determined following the method of Jerves-Andrade et al.[23] Briefly, the extract was analysed at decreasing concentrations of 64, 16, 4, 1 and 0.25 μg mL^{-1} in a 96-well plate. The culture medium was TSB, and the plates were inoculated for 24 h at 37 °C. Ampicillin (Genfar, Colombia) was used as a positive control of the assay. After 24 hours of incubation, the absorbance of each well of the plates was measured with an ELISA microplate reader (Multiskan-EX, Thermo Scientific, Shanghai, China) at 405 nm. Growth-inhibitory activity was expressed as a mean percentage of growth inhibition with respect to a control without antibacterial compound. Negligible antibacterial effects were considered when the growth inhibition percentage was lower than 25% at the maximum concentration tested.

Anti-inflammatory capacity

Extract preparation

The aqueous and hydroalcoholic extracts of young guayusa leaves were prepared under the same conditions that were used for the determination of antibacterial activity. After that, the extracts were freeze-dried and then reconstituted with methanol up to a concentration of 20 mg mL^{-1}. The mixture was shaken for 24 h with a rotary stirrer. Finally, the extracts were sonicated at 5 °C during 1 h just before the assay.

Hyaluronidase inhibition

The protocol proposed by Sigma Aldrich[24] was used to assess the inhibitory effect of guayusa extracts against hyaluronidase. The effect was evaluated at decreasing concentrations of the extracts (50, 25, 12.5, 6.25, 3.125 and 1.562 μg mL^{-1}). The analysis was performed with 1.6 enzyme units 100 μL^{-1} of phosphate buffer (300 mM). Five μL of each dilution was transferred to a well-plate, mixed with 66.5 μL of enzyme diluent solution (20 mM sodium phosphate with 77 mM sodium chloride and 0.01% (w/v) bovine serum albumin, pH 7.0) and 33.5 μL enzyme solution. The ensemble was

homogenized and incubated at 37 °C for 10 min. After, 100 µL hyaluronic acid solution (0.3 mg mL^{-1} in phosphate buffer with hyaluronic acid) was added and then it was incubated for 45 min at 37 °C. Finally, 50 µL of each concentration was taken and mixed with 250 µL of acidic albumin solution (24 mM sodium acetate, 79 mM acetic acid with 0.1% (w/v) bovine serum albumin, pH 3.75). It was homogenized and, after 10 min at room temperature, the absorbance at 600 nm was measured. All assays were performed in quadruplicate and compared with a standard of indomethacin (Sigma®) and acetylsalicylic acid (Merck®) at a concentration of 20 mg mL^{-1} dissolved with a 7.5% sodium bicarbonate solution. The percentage of inhibition was calculated by the ratio between the absorbance of the test with the extract at 600 nm and the absorbance of the maximum inhibition at 600 nm (Blank).

Statistical analysis

The data obtained were expressed as mean values (n = 3) ± standard deviation (SD). All values were subjected to analysis of variance (ANOVA) with a 95% confidence level. Pearson's correlation analysis was performed to corroborate the relationships between bioactive compounds (polyphenols and carotenoids) and antioxidant capacity, with a 95 % confidence level. All statistical tests were conducted using STATGRAPHICS Centurion version XVI.I (Statgraphics.Net, Madrid, Spain).

RESULTS AND DISCUSSION

Phenolic composition

The identified phenolic compounds in guayusa leaves by HPLC-DAD-ESI-MS^n are presented in Table 1. Phenolic compounds in guayusa have been previously described by our research group in García-Ruiz et al.[25] A total of 14 phenolic compounds were

identified and quantified in both maturity stages. Nine compounds corresponded to hydroxycinnamic acids and derivatives, and 5 compounds were flavonoids. The phenolic compounds identified in guayusa leaves have been previously described in other species of the genus *Ilex*, such as *Ilex paraguariensis*[26], *Ilex latifolia*[27] and *Ilex kudingcha*[28].

The hydroxycinnamic acid derivatives were the major constituents of the phenolic fraction, representing 61% and 78% of total polyphenols in young and old leaves, respectively (Table 2). Chlorogenic acid was the main phenolic compound found in both types of guayusa leaves, being its concentration higher in young leaves (7.63 g Kg^{-1} DW) than in old leaves (6.51 g Kg^{-1} DW). Clifford and Ramirez-Martinez[29] also described the chlorogenic acid as the principal constituent of the phenolic fraction in mate leaves. Numerous reports have stated the biological effects of chlorogenic acid, such as high antioxidant capacity, hepato-protective, hypoglycemic and antiviral activities, among others. [30]

With regard to flavonols, their total content evaluated as the sum of the content of individual flavonols was 3.16 g Kg^{-1} in young leaves and 1.98 g Kg^{-1} in old leaves. Quercetin-3-*O*-hexose was the main flavonol in young and old leaves with a concentration of 2.46 and 1.63 g Kg^{-1}, respectively. Quercetin has shown a variety of biological activities, such as ROS scavenging, a protective role in auditory function and a reduction in apoptotic cells when was administered together with gentamicin; besides stimulation of insulin secretion via a mechanism of action based on an increase in intracellular calcium.[31]

As shown in Table 2, TPC of young leaves was 1.6-fold higher than old leaves. This result suggests that the maturity stage could influence on the TPC in guayusa leaves. Unripe plant organisms are characterized by having a higher rate of metabolite

biosynthesis in young tissues than in the old ones.[32] Hence, young plant tissues have higher phenolic contents since they act as chemical deterrents for herbivory and pests, thus protecting young vegetables and allowing their maturation and reproduction. Moreover, phenolic compounds are also responsible for protecting the plant from damaging radiations. [33]

Carotenoids content

As secondary metabolites, the content and types of carotenoids in plants depend on several pre and post harvesting factors, genotype, ripening time, cultivation method and climatic conditions, processing. [34] As shown in Table 3, a total of seven carotenoids were identified in both maturity stages. In young leaves, the sum of the quantified carotenoids was 44 μg g^{-1} DW, while in old leaves was 39 μg g^{-1} DW. A part of lutein, α and β-carotenes are also in relative high concentration in guayusa leaves. Rodriguez-Amaya et al.[35], explain that green vegetables have a defined qualitative pattern with lutein, β-carotene, violaxanthin and neoxanthin as the principal carotenoids. The relative proportions of these carotenoids are fairly constant, but the absolute concentrations vary considerably.

Lutein was the main carotenoid with a concentration of 25.72 μg g^{-1} DW in young leaves and 22.33 μg g^{-1} DW in old leaves. Lutein is a pigment related with the process of photosynthesis [36], and this may explain why this is the principal carotenoid on guayusa leaves.

Lutein is found together with zeaxanthin in the retina, apart from other tissues. Even lutein has no provitamin-A activity in humans, its biological activities have attracted great attention in relation to human health. The presence of this compound in the retinal tissue has been reported to reduce longitudinal chromatic aberration, as well as protect against cataract and age-related macular degeneration.[37] Apart from the possible

beneficial activity of lutein on vision, Schünemann et al.[38], reported that it is strongly positive associated with pulmonary function.

Ripening allows the production of carotenoids [35], which could explain why certain carotenoids (Table 3) are found in higher quantity in old leaves. At the same time, ripening favours the degradation of chloroplasts and their photosynthetic structures, together with the disappearance of chlorophylls and the characteristic carotenoids of the chloroplasts, β - carotene, lutein and violaxanthin[39], which explains the decrease of these carotenoids in guayusa leaves with maturation.

Antioxidant capacity

Plants represent a potential source of several phytochemicals, such as polyphenols and carotenoids, with relevant antioxidant capacities, which were determined in guayusa leaves by $DPPH^{\cdot}$ and ORAC methods (Table 4). In general, guayusa showed the ability of scavenging $DPPH^{\cdot}$ and ORAC radicals at various degrees in both maturity stages; however, when this function was measured by the $DPPH^{\cdot}$ method, the overall trend of antioxidant capacity followed the concentration of phenolic compounds (Table 2). The young leaves displayed significant higher antioxidant capacity (16.19 mmol Trolox $100g^{-1}$ DW). In contrast, the ripening state did not show any influence on the antioxidant capacity when it was measured by the ORAC method, but it was significantly higher (≈ 65 mmol Trolox $100g^{-1}$ DW) than the results obtained with the $DPPH^{\cdot}$ method. This may be due to the fact that ORAC method is based on hydrogen atom transfer; in consequence, guayusa leaves possess a higher peroxyl radical scavenging capacity.

Antioxidant capacities obtained above must comprise a mixture of both polar and non-polar compounds. As shown in Table 5, polyphenols and carotenoids showed a positive correlation with the antioxidant capacity. Moreover, phenolic compounds where in

higher concentration than carotenoids (Tables 2 and 3) in both maturity stages, which could suggest that phenolic compounds might be involved in the high antioxidant capacity of the guayusa leaves; and particularly hydroxycinnamic acid derivatives present in major quantities in both maturity stages of the guayusa tested. Using the same $DPPH^{\cdot}$ radical methodology, Bassani et al.[40], demonstrated a direct positive correlation between antioxidant activity and phenolic compounds in yerba mate.

In vitro digestibility

This *in vitro* static digestion model allowed the estimation of the bioaccessibility of dietary polyphenols after each digestion phase, as well as their antioxidant capacity. As shown in Table 6, TPC decreased approximately a 59% during *in vitro* digestion process. This could be due to the sensibility of polyphenols to the changes of pH that is produced during digestion. The diminution of TPC on the oral-simulated digestion of guayusa infusion (1.08%) is in agreement with studies that have shown that most dietary polyphenols appear to be quite stable during oral-simulated digestion.[41]

The low pH and the presence of enzymes were the main difference between oral and stomachic phase, in which a drop of TPC was observed until 50% in comparison with the initial value. The low pH could be the responsible of the considerably diminution of TPC, which also might affect its biological activities. Although enzymes were part of the system, Silberberg et al.,[42] explain that enzymes did not exert a negative function on the phenolic structures.

In the last simulated phases, when the pH was alkalinized, the phenolic compounds decreased until they reach the lowest concentration, representing the 45% of the initial content. In this phase, the drastic variation in the chemical conditions and high pH could affect to the stability of some phenolic acids[43], which could explain this reduction.

In vitro antioxidant assays are very useful for preliminary screening of the antioxidant potential of natural products, allowing better-informed food product design and useful interventions to improve consumer health. During *in vitro* gastrointestinal digestion, antioxidant capacity measured by $ABTS^{\cdot+}$ method significantly decreased (Table 6). It may be possible that the pH could exert an effect of the structure of phenolic compounds along the digestion, which also may affect them antioxidant capacity.[44, 45] It seems that this characteristic is mainly attributed to the particular structures and functional groups of phenolic compounds, and not so much to the total amounts of these components. This behavior is corroborated by Tagliazucchi et al.,[46] who demonstrated the reduction of $ABTS^{\cdot+}$ radical scavenging related with phenolic composition of food matrices during gastric digestion. In this study, the 5-*O*-Caffeoylquinic acid was the main phenolic compound quantified in guayusa leaves (Table 2), and according to Daglia et al.[47], it is the most active fraction against free radicals, whose reduction may explain the drop of the antioxidant capacity. Furthermore, chlorogenic acid isomers, the major constituents of phenolic fraction in guayusa leaves, have proven to be very unstable after digestion[48], being almost degraded in aqueous infusions.[49]

Antibacterial activity

In the present study, the inhibitory effects of aqueous and hydroalcoholic guayusa extracts against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were also evaluated. The results revealed that guayusa extracts did not exhibit any antibacterial effect for both strains (results not shown), which agreed with previous results reported for yerba mate.[50]

Anti-inflammatory activity

As shown in Table 7, aqueous and hydroalcoholic guayusa extracts displayed an anti-inflammatory activity. Particularly, this activity was > 66% in both extracts, showing

the aqueous extract the highest inhibitory effect (131.70%). Characterization of *Ilex* species have shown the presence of several polysaccharides and relative higher contents of sulfuric acid.[51] Wolf et al.[52], demonstrated that sulfated oligosaccharides can inhibit hyaluronidase by a competitive mechanism. In another study, Katsube et al.[53], isolated a polysaccharide from hot water extract of sporophyll of *Undaria pinnatifida*. The isolated polysaccharide was found to contain sulfuric acid and sugars such as L-fructose, D-galactose, and D-glucuronic acids. The sulfated polysaccharide showed inhibitory activity against hyaluronidase in a dose dependent manner. Therefore, it is possible that a group of (sulfated) polysaccharides extracted from guayusa leaves could be the responsible for the inhibition of hyaluronidase.

On the other hand, it is important to emphasize that anti-inflammatory activity of guayusa extracts was higher than the activity exhibited by acetylsalicylic acid and indomethacin, individually known for their anti-inflammatory activity.[54, 55]

CONCLUSION

Guayusa ripening stage significantly influenced the content of phenolic compounds and antioxidant capacity (measured by $DPPH^{\cdot}$ method), with a decrease in mature leaves over the younger ones, but not in the content of carotenoids. On the other hand, during the *in vitro* gastrointestinal digestion, the TPC and antioxidant capacity of the guayusa infusion diminished, which could be due to the influence of pH conditions on the stability of phenolic compounds. Aqueous and hydroalcoholic guayusa extracts did not show any antibacterial effects against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, but they exhibited a strong anti-inflammatory activity by inhibiting hyalurodinase. Overall, the results show that young leaves of guayusa could be used as a food

ingredient as well as for the development of new anti-inflammatory therapies and botanical formulations. Further investigation is necessary to evaluate the bioavailability of phenolic compounds individually after the digestion process, as well as the characterization of the anti-inflammatory active compounds in preclinical and clinical models.

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REFERENCES

1. Slavin J and Lloyd B, Health Benefits of Fruits and Vegetables. *Adv Nutr* **3**:506-516 (2012).

2. Giovannini P, Medicinal plants of the Achuar (Jivaro) of Amazonian Ecuador: Ethnobotanical survey and comparison with other Amazonian pharmacopoeias. *Journal of ethnopharmacology* **164**:78-88 (2015).

3. Bennett BC, Plants and People of the Amazonian Rainforests. *BioScience* **42**:599-607 (1992).

4. Del Rio D, Rodríguez-Mateos A, Spencer J, Tognolini M, Borges G and Crozier A, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid Redox Signal* **18**:1818-1892 (2013).

5. Filip R, López P, Giberti G, Coussio J and Ferraro G, Phenolic compounds in seven South American *Ilex* species. *Fitoterapia* **72**:774-778 (2001).

6. Gil-Chávez G, Villa J, Ayala-Zavala F, Heredia J, Sepulveda D, Yahia E and González-Aguilar A, Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview. *Compr Rev Food Sci Food Saf* **12**:5-23 (2012).

7. Yi F, Zhao X, Peng Y and Xiao P, Genus *Ilex* L.: Phytochemistry, Ethnopharmacology, and Pharmacology. *Chinese Herbal Medicines* **8**:209-230 (2016).

8. Puerta-Gomez AF and Cisneros-Zevallos L, Postharvest studies beyond fresh market eating quality: Phytochemical antioxidant changes in peach and plum fruit during ripening and advanced senescence. *Postharvest Biol Technol* **60**:220-224 (2011).

9. Blum-Silva C, Chaves V, Schenkel E, Coelho G and Reginatto F, The influence of leaf age on methylxanthines, total phenolic content, and free radical scavenging capacity of *Ilex paraguariensis* aqueous extracts. *Rev Bras Farmacogn* **25**:1-6 (2015).

10. Torres G, *El aprovechamiento de la Guayusa (Ilex guayusa): Manual de buenas prácticas de recolección para la cosecha de hojas*. Fundación Chankuap, Ecuador (2013).

11. Crespo Coello P, La Guayusa: Trayectoria y Sentido, in *Programa de Manejo Forestal Sostenible en la Región Andina*, Ed. IICA - Ministerio de Asuntos Exteriores de Finlandia (2013).

12. Meléndez-Martínez AJ, Britton G, Vicario IM and Heredia FJ, Relationship between the colour and the chemical structure of carotenoid pigments. *Food chemistry* **101**:1145-1150 (2007).

13. Slinkard K and Singleton VL, Total phenol analysis: Automation and comparison with manual methods. *Am J Enol Vitic* **28**:49-55 (1977).

14. Gironés-Vilaplana A, Baenas N, Villaño D, Speisky H, Garcíaa-Viguera C and Moreno D, Evaluation of Latin-American fruits rich in phytochemicals with biological effects. *J Funct Foods* **7**:599–608 (2014).

15. Stinco CM, Benítez-González AM, Hernanz D, Vicario IM and Meléndez-Martínez AJ, Development and validation of a rapid resolution liquid chromatography method for the screening of dietary plant isoprenoids: Carotenoids, tocopherols and chlorophylls. *J Chromatogr A* **1370**:162-170 (2014).

16. Mena P, García-Viguera C, Navarro-Rico J, Moreno D, Bartual J, Saura D and Martí N, Phytochemical characterisation for industrial use of pomegranate *(Punica granatum* L.) cultivars grown in Spain. *J Sci Food Agric* **91**:1893–1906 (2011).

17. Ou B, Hampsch-Woodill M and Prior R, Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. *J Agric Food Chem* **49**:4619-4626 (2001).

18. Re R, Pellegrini R, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decoloration assay. *Free Radic Biol Med* **26**:1231–1237 (1999).

19. Hartwig VG, Brumovsky LA and Fretes MR, A Total Polyphenol Content of Mate (*Ilex paraguariensis*) and Other Plants-derived Beverages. *JFR* **1**:58-67 (2012).

20. Tavares L, Figueira I, Macedo D, McDougall G, Leitao M, Vieira H, Stewart D, Alves P, Ferreira R and Santos C, Neuroprotective effect of blackberry (*Rubus* sp.) polyphenols is potentiated after simulated gastrointestinal digestion. *Food chemistry* **131**:1443-1452 (2012).

21. Ryan L, O'Connell O, O'Sullivan L, Atherne S and O'Brien N, Micellarisation of Carotenoids from Raw and Cooked Vegetables. *Plant Foods Hum Nutr* **63**:127–133 (2008).

22. Guendouze-Bouchefa N, Madani K, Chibane M, Boulekbache-Makhlouf L, Hauchard D, Kiendrebeogo M, Stévigny C, Okusa PN and Duez P, Phenolic compounds, antioxidant and antibacterial activities of three *Ericaceae* from Algeria. *Ind Crops Prod* **70**:459-466 (2015).

23. Jerves-Andrade L, León-Tamariz F, Peñaherrera E, Cuzco N, Tobar V, Ansaloni R, Maes L and Wilches I, Medicinal plants used in South Ecuador for gastrointestinal problems: An evaluation of their antibacterial potential. *J Med Plants Res* **8**:1310-1320 (2014).

24. Sigma-Aldrich, *Enzymatic Assay of Hyaluronidase*. http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-hyaluronidase.html [13/02 2017].

25. García-Ruiz A, Baenas N, Benítez-González AM, Stinco CM, Meléndez-Martínez A, Moreno D and Ruales J, Guayusa (*Ilex guayusa* L.) new tea: phenolic and carotenoid composition and antioxidant capacity. *J Sci Food Agric* **97**:3929–3936 (2017).

26. Bravo L, Goya L and Lecumberri E, LC/MS characterization of phenolic constituents of mate (*Ilex paraguariensis*, St. Hil.) and its antioxidant activity compared to commonly consumed beverages. *Food Res Int* **40**:393-405 (2007).

27. Fan C, Deng J, Yang Y, Liu J, Wang Y, Zhang X, Fai K, Zhang Q and Ye W, Multi-ingredients determination and fingerprint analysis of leaves from *Ilex latifolia* using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *Journal of pharmaceutical and biomedical analysis* **84**:20-29 (2013).

28. Zhu F, Cai Y, Sun M, Ke J, Lu D and Corke H, Comparison of Major Phenolic Constituents and *in Vitro* Antioxidant Activity of Diverse Kudingcha Genotypes from *Ilex kudingcha*, *Ilex cornuta*, and *Ligustrum robustum*. *J Agric Food Chem* **57**:6082–6089 (2009).

29. Clifford MN and Ramirez-Martinez JR, Chlorogenic acids and purine alkaloids contents of Maté (*Ilex paraguariensis*) leaf and beverage. *Food chemistry* **35**:13-21 (1990).

30. Sanchez MB, Miranda-Perez E, Verjan JCG, de los Angeles Fortis Barrera M, Perez-Ramos J and Alarcon-Aguilar FJ, Potential of the chlorogenic acid as multitarget agent: Insulin-secretagogue and PPAR α/γ dual agonist. *Biomedicine & Pharmacotherapy* **94**:169-175 (2017).

31. Boots A, Haenen G and Bast A, Health effects of quercetin: from antioxidant to nutraceutical. *Eur J Pharmacol* **585**:325-337 (2008).

32. Hartmann T, Diversity and variability of plant secondary metabolism: a mechanistic view *Entomol Exp Appl* **80**:177-188 (1996).

33. Di Ferdinando M, Brunetti C, Agati G and Tattini M, Multiple functions of polyphenols in plants inhabiting unfavorable Mediterranean areas. *Environmental and Experimental Botany* **103**:107-116 (2014).

34. Saini R, Nile S and Park S, Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. *Food Research International* **76**:735-750 (2015).

35. Rodriguez-Amaya DB, Kimura M, Godoy HT and Amaya-Farfan J, Updated Brazilian database on food carotenoids: Factors affecting carotenoid composition. *Journal of Food Composition and Analysis* **21**:445-463 (2008).

36. Mathis P and Schenck C, The functions of carotenoids in photosynthesis, in *Carotenoid chemistry and biochemistry*, ed. by Britton G and Goodwin T. IUPAC - Pergamon Press, UK, pp 339-482 (1982).

37. Madaan T, Choudhary AN, Gyenwalee S, Thomas S, Mishra H, Tariq M, Vohora D and Talegaonkar S, Lutein, a versatile phyto-nutraceutical: An insight on pharmacology, therapeutic indications, challenges and recent advances in drug delivery. *PharmaNutrition* **5**:64-75 (2017).

38. Schünemann H, McCann S, Grant B, Trevisan M, Muti P and Freudenheim J, Lung Function in Relation to Intake of Carotenoids and Other Antioxidant Vitamins in a Population-based Study. *Am J Epidemiol* **155**:463-471 (2002).

39. Arscott S, Food sources of Carotenoids, in *Carotenoids and Human Health*, ed. by Tanumihardjo S. Humana Press, USA, pp 3-19 (2013).

40. Bassani D, Nunes D and Granato D, Optimization of Phenolics and Flavonoids Extraction Conditions and Antioxidant Activity of Roasted Yerba-Mate Leaves (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) using Response Surface Methodology. *Anais da Academia Brasileira de Ciências* **86**:923-933 (2014).

41. Pinacho R, Cavero RY, Astiasarán I, Ansorena D and Calvo MI, Phenolic compounds of blackthorn (*Prunus spinosa* L.) and influence of *in vitro* digestion on their antioxidant capacity. *J Funct Foods* **19, Part A**:49-62 (2015).

42. Silberberg M, Morand C, Mathevon T, Besson C, Manach C, Scalbert A and Remesy C, The bioavailability of polyphenols is highly governed by the capacity of the intestine and of the liver to secrete conjugated metabolites. *Eur J Nutr* **45**:88-96 (2006).

43. Friedman M and Jurgens H, Effect of pH on the Stability of Plant Phenolic Compounds. *J Agric Food Chem* **48**:2101-2110 (2000).

44. Kosińska A, Xie Y, Diering S, Héritier J and Andlauer W, Stability of Phenolic Compounds Isolated from Cocoa, Green Tea and Strawberries in Hank's Balanced Salt Solution under Cell Culture Conditions. *Pol J Food Nutr Sci* **62**:91-96 (2012).

45. Gullon B, Pintado ME, Fernández-López J, Pérez-Álvarez JA and Viuda-Martos M, In vitro gastrointestinal digestion of pomegranate peel (Punica granatum) flour obtained from co-products: Changes in the antioxidant potential and bioactive compounds stability. *Journal of Functional Foods* **19**:617-628 (2015).

46. Tagliazucchi D, Verzelloni E, Bertolini D and Conte A, *In vitro* bio-accessibility and antioxidant activity of grape polyphenols. *Food chemistry* **120**:599-606 (2010).

47. Daglia M, Racchi M, Papetti A, Lanni C, Govoni S and Gazzani G, *In Vitro* and *ex Vivo* Antihydroxyl Radical Activity of Green and Roasted Coffee. *Journal of agricultural and food chemistry* **52**:1700-1704 (2004).

48. Boaventura BCB, Amboni RDdMC, da Silva EL, Prudencio ES, Di Pietro PF, Malta LG, Polinati RM and Liu RH, Effect of *in vitro* digestion of yerba mate (*Ilex paraguariensis* A. St. Hil.) extract on the cellular antioxidant activity, antiproliferative activity and cytotoxicity toward HepG2 cells. *Food Research International* **77, Part 2**:257-263 (2015).

49. Siracusa L, Kulisic-Bilusic T, Politeo O, Krause I, Dejanovic B and Ruberto G, Phenolic Composition and Antioxidant Activity of Aqueous Infusions from *Capparis spinosa* L. and *Crithmum maritimum* L. before and after Submission to a Two-Step *in Vitro* Digestion Model. *Journal of agricultural and food chemistry* **59**:12453–12459 (2011).

50. Prado Martin JG, Porto E, de Alencar SM, da Glória EM, Corrêa CB and Ribeiro Cabral IS, Antimicrobial activity of yerba mate (*Ilex paraguariensis* St. Hil.) against food pathogens. *Rev Argent Microbiol* **45**:93-98 (2013).

51. Fan J, Wu Z, Zhao T, Sun Y, Ye H, Xu R and Zeng X, Characterization, antioxidant and hepatoprotective activities of polysaccharides from Ilex latifolia Thunb. *Carbohydrate Polymers* **101**:990-997 (2014).

52. Wolf RA, Glogar D, Chaung L-Y, Garrett PE, Ertl G, Tumas J, Braunwald E, Kloner RA, Feldstein ML and Muller JE, Heparin inhibits bovine testicular hyaluronidase activity in myocardium of dogs with coronary artery occlusion. *Am J Cardiol* **53**:941-944 (1984).

53. Katsube T, Yamasaki Y, Iwamoto M and Oka S, Hyaluronidase-Inhibiting Polysaccharide Isolated and Purified from Hot Water Extract of Sporophyll of *Undaria pinnatifida*. *Food Sci Technol Res* **9**:25-29 (2003).

54. Khan SA, Chatterjee SS and Kumar V, Low dose aspirin like analgesic and anti-inflammatory activities of mono-hydroxybenzoic acids in stressed rodents. *Life Sciences* **148**:53-62 (2016).

55. Kwasigroch B, Escribano E, Morán MdC, Queralt J, Busquets MA and Estelrich J, Oil-in-water nanoemulsions are suitable for carrying hydrophobic compounds: Indomethacin as a model of anti-inflammatory drug. *Int J Pharm* **515**:749-756 (2016).

Table 1. HPLC – DAD – ESI/MS^n data for the phenolic composition compounds from guayusa leaves

Peak	Rt (min)	M^-	MS2 fragments	Phenolic Compounds
1	6.0	707*, 353	191 (100), 179 (42.6)**	3-*O*-CQA (Neochlorogenic acid)
2	6.6	341	179 (63.0), 135 (10.6)	Caffeoyl-hexose
3	8.2	341	179 (100), 135 (10.4)	Caffeoyl-hexose
4	9.6	341	179 (78.9), 135 (4.9)	Caffeoyl-hexose
5	10.6	353	191 (100), 179 (4.7)	5-*O*-CQA (Chlorogenic acid)
6	12.5	707*, 353	191 (100), 179 (3.2)	5-*O*-CQA isomer
7	20.7	367	193 (51.3), 173 (0.8)	3-Feruloylquinic acid
8	27.3	609	301 (100), 271 (23.1)	Quercetin-3-*O*-rutinoside (Rutin)
9	28.5	609	301 (100), 271 (15.2)	Rutin Isomer
10	32.5	463	301 (100)	Quercetin-3-*O*-hexose
11	34.3	463	301 (100)	Quercetin-3-*O*-hexose
12	36.1	515	353 (100), 191 (9.7)†, 179 (1.3)	3,5-Dicaffeoylquinic acid (Isochlorogenic acid)
13	42.8	447	285 (100)	Kaempferol-3-*O*-hexose
14	43.7	1031*, 515	353 (61.4), 173 (10.43)†	3,4-Dicaffeoylquinic acid

Rt: retention time, * Dimeric adduct, **Relative abundance (software ca. %); †Base Peak in MS2 (MS3 100% Rel. abun.). CQA (Caffeoylquinic acid)

Table 2. Phenolic composition in guayusa leaves influenced by ripening

	Concentration (g Kg^{-1} DW)	
	Young leaves	**Old leaves**
Total phenolics content*	33.44 ± 0.48[a]	21.42 ± 0.87[b]
Phenolic compounds		
3-*O*-Caffeoylquinic acid	4.10 ± 0.29[a]	3.66 ± 0.29[a]
Caffeoyl-hexose	0.13 ± 0.01[a]	0.08 ±0.01[b]
Caffeoyl-hexose	0.04 ± 0.01[a]	0.03 ± 0.00a
Caffeoyl-hexose	0.06 ±0.00[a]	0.02 ± 0.02[b]
5-*O*-Caffeoylquinic acid	7.63 ±0.54[a]	6.51 ± 0.57[a]
5-*O*-Caffeoylquinic acid isomer	0.07 ±0.00[a]	0.05 ± 0.01[b]
Feruloylquinic acid	0.08 ± 0.00[a]	0.06 ± 0.00[b]
Quercetin-3-*O*-rutinoside (Rutin)	0.15 ± 0.00[a]	0.07 ± 0.01[b]
Rutin isomer	0.26 ± 0.01[a]	0.15 ± 0.02[b]
Quercetin-3-*O*-hexose	1.01 ± 0.07[a]	0.71 ± 0.08[b]
Quercetin-3-*O*-hexose	1.45 ± 0.08[a]	0.92 ± 0.06[b]
3,5, Dicaffeoylquinic acid	7.12 ± 0.48[a]	5.37 ± 0.43[b]
Kaempferol-3-*O*-hexose	0.29 ± 0.01[a]	0.13 ± 0.03[b]
3,4, Dicaffeoylquinic acid	1.12 ± 0.09[a]	0.93 ± 0.08[b]

* Total phenolics content, by Slinkard and Singleton method, is expressed as mg of gallic acid equivalents per g.

a – b Mean values with different letter on the right in the same row indicate statistically significant differences among the maturity stage of the leaves ($p<0.05$).

(n = 3)

Table 3. Carotenoid composition in guayusa leaves influenced by ripening

	Concentration (μg g^{-1} DW)	
	Young leaves	**Old leaves**
Total carotenoids*	44.00 ± 8.88[a]	38.92 ± 7.62[a]
Xanthophyll		
Neoxanthin	1.79 ± 0.59[a]	1.94 ± 0.42[a]
Violaxanthin	2.13 ± 0.78[a]	1.75 ± 0.37[a]
Lutein	25.72 ± 6.46[a]	22.33 ± 2.93[a]
Carotenes		
α – carotene	7.76 ± 1.34[a]	6.35 ± 0.56[a]
cis α – carotene	2.01 ± 0.76[a]	2.38 ± 0.01[a]
β – carotene	4.06 ± 1.06[a]	3.65 ± 0.49[a]
cis β – carotene	0.55 ± 0.13[a]	0.52 ± 0.04[a]

*Total carotenoids, by RRLC, is expressed as μg per g.

a Mean values with same letter on the right in the same row indicate no statistically significant differences among the maturity stage of the leaves ($p < 0,05$).

Table 4. Antioxidant capacity in guayusa leaves influenced by ripening

	Antioxidant capacity (mmol Trolox 100g^{-1} DW)	
	Young leaves	***Old leaves***
DPPH	16.19 ± 0.37[a]	12.47 ± 0.85[b]
ORAC	69.88 ± 7.49[a]	61.38 ± 6.55[a]

a – b Mean values with different letter on the right in the same row indicate statistically significant differences among the maturity stage of the leaves ($p<0.05$).

Table 5. Pearson's correlation coefficients (*r*) between bioactive compounds (polyphenols and carotenoids) of young and old guayusa leaves and its antioxidant capacity ($DPPH^{\cdot}$ and ORAC).

	Samples	
	Young leaves	**Old leaves**
$DPPH^{\cdot}$	1.00	1.00
ORAC	1.00	1.00

Significant at $p < 0.05$

Table 6. Effect of simulated gastrointestinal process on the phenolic composition of guayusa infusion and their antioxidant capacity at the beginning and after the final step

Gastrointestinal phase	**Total phenolics content*** (g GAE Kg^{-1} DW)	**Antioxidant capacity** (mmol Trolox g^{-1} DW)$^{\Omega}$
Initial	7.12 ± 1.02^{a}	1.8 ± 0.86^{a}
Oral	7.04 ± 0.21^{a}	
Stomachic	3.67 ± 0.32^{b}	
Intestinal	3.22 ± 0.41^{b}	
Final	2.92 ± 0.10^{b}	0.21 ± 0.017^{b}

* mg of gallic acid equivalents per g of dry weight.
$^{\Omega}$ Antioxidant capacity, by $ABTS^{\cdot+}$ method is expressed as mmol Trolox per g.
a – b Mean values with different letter on the right in the same row indicate statistically significant differences among the different phases of in vitro gastrointestinal digestion ($p<0.05$).

Table 7. Percentage of hyaluronidase inhibition with guayusa young leaves extracts and commonly used anti-inflammatory drugs

	Guayusa extract		**Controls**	
	Hydroalcoholic	***Aqueous***	***Acetylsalicylic acid***	***Indomethacin***
50µg/mL	66.82 ± 7.75^{a}	131.70 ± 6.62^{b}	54.60 ± 1.70^{b}	74.66 ± 5.34^{c}
25µg/mL	62.95 ± 9.77^{a}	95.52 ± 12.28^{b}	58.32 ± 2.90^{a}	67.76 ± 0.67^{a}
12.5µg/mL	56.30 ± 3.51^{c}	83.13 ± 3.84^{a}	52.00 ± 7.89^{c}	66.23 ± 3.77^{b}
6.25µg/mL	55.13 ± 3.44^{bc}	68.38 ± 0.45^{a}	51.91 ± 7.63^{c}	63.16 ± 2.02^{ab}
3.125µg/mL	52.87 ± 1.13^{b}	63.65 ± 2.43^{a}	55.30 ± 1.90^{b}	61.62 ± 3.98^{a}
1.562µg/mL	49.48 ± 1.06^{a}	58.64 ± 5.19^{a}	52.82 ± 6.94^{a}	60.93 ± 9.76^{a}

a – c Mean values with different letter on the right in the same row indicate statistically significant differences among the tested anti-inflammatories ($p<0.05$)